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Differing roles of CD18 and VLA-4 in leukocyte migration/activation during anti-GBM nephritis

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Differing roles of CD18 and VLA-4 in leukocyte migration/activation during anti-GBM nephritis. The mechanisms underlying leukocyte migration into inflamed glomeruli and their *in situ* activation are incompletely understood. We addressed this issue by investigating the effects of monoclonal antibodies (mAbs) to CD18 and VLA-4 on these process in the heterologous phase of anti-glomerular basement membrane (GBM) nephritis in rat. Anti-CD18 mAb substantially attenuated neutrophil (PMN) migration into glomeruli and the accompanying proteinuria which is a function of *in situ* leukocyte activation (ca. 60%). Anti-VLA-4 mAb modestly inhibited PMN migration (ca. 20%) and had no significant effect on proteinuria. Combination of both mAbs was no more effective than anti-CD18 mAb alone. Despite continued mAb blockade of CD18 or VLA-4 (or both), macrophage (Mϕ) migration following PMN influx was unaltered. However, combined CD18/VLA-4 mAbs diminished the proteinuria associated with Mϕ influx (ca. 50%). Abrogation of the acute influx of PMNs in this model (via complement depletion or anti-PMN antibody) did not diminish the following influx of Mϕs, although the associated proteinuria was abolished. In this context, Mϕ migration was substantially decreased by anti-VLA-4 mAb (ca. 50%), but not anti-CD18 mAb (either alone or with anti-VLA-4 mAb). In sum, leukocyte migration and activation in the heterologous phase of anti-GBM nephritis are dependent on CD18 and VLA-4, although to varying degrees depending on the leukocyte subtype and the presence of prior inflammation. Nonetheless, a significant component of both PMN and Mϕ migration/activation is CD18/VLA-4 independent.

Leukocytes are critical effectors of glomerular damage in the context of immune-mediated glomerulonephritis. They can induce directly acute glomerular dysfunction via their elaboration of reactive oxygen species and proteases, influence the evolution of the inflammatory process via their elaboration of cytokines, and ultimately play an important role in determining whether acute glomerular injury resolves or produces glomerulosclerosis [1–4]. Given their pivotal nature as effectors of glomerular injury and scarring, understanding the mechanisms underlying their influx into the inflamed glomerulus is critically important.

The influx of leukocytes into a focus of inflammation and *in situ* activation are both a complex function of cytokines, chemokines and adhesion molecules [5, 6]. With respect to adhesion molecules, it has become apparent from *in vitro* and *in vivo* data that CD18 and VLA-4 are the principal leukocyte adhesion molecules which regulate the adherence and migration of neutrophils (PMNs) and macrophages (Mϕs) during inflammation, although the contribution of these adhesion molecules may vary considerably as a function of the tissue and inflammatory stimulus [7–15].

Apropos to this issue, a number of recent studies have explored the role of leukocyte adhesion molecules in mediating the influx and *in situ* activation of leukocytes in anti-glomerular basement membrane (GBM) nephritis although not with entirely consonant results. Two recent studies found that CD18 is the predominant leukocyte adhesion molecule which mediates the influx of PMNs into the glomerulus and their *in situ* activation [16, 17], although another recent study failed to discern a role for CD18 [18]. In addition, recent data suggest that VLA-4 (CD49d/CD29) participates in the acute influx of PMNs in anti-GBM nephritis [17], although the relative contribution of this adhesion molecule is uncertain.

Little is known about the leukocyte adhesion molecules underlying the influx of Mϕs into the glomerulus in the context of anti-GBM nephritis, although it has been clear for some time that the influx/activation of PMNs and Mϕs are not obligatorily linked processes [19, 20]. Recent studies have suggested that CD11a/CD18 is necessary for the migration of monocytes into the glomerulus and chronic glomerular injury [21, 22]. These studies, however, contrast with other studies of inflammation which have found that CD18 *per se* mediates only a small portion of monocyte migration, and that VLA-4 provides a critical CD18-independent pathway of monocyte adhesion and migration [7–13].

To address these uncertainties, we examined the independent and combined roles of CD18 and VLA-4 in the migration of leukocytes (both PMNs and monocytes/Mϕs) into the glomerulus during the heterologous phase of anti-GBM nephritis using *in vivo* monoclonal (mAb) blockade of these adhesion molecules. Because migration and *in situ* activation are separable phenomena [16, 23], we additionally sought to determine the role of these adhesion receptors in the activation of glomerular leukocytes as manifested by proteinuria. Our prima facie hypothesis was that both integrins would be important for leukocyte migration/activation, although their relative importance would differ for PMNs and monocytes. Although the data we derived in the current work support this hypothesis, they also suggest that a substantial component of leukocyte migration/activation is CD18/VLA-4-independent in this model.

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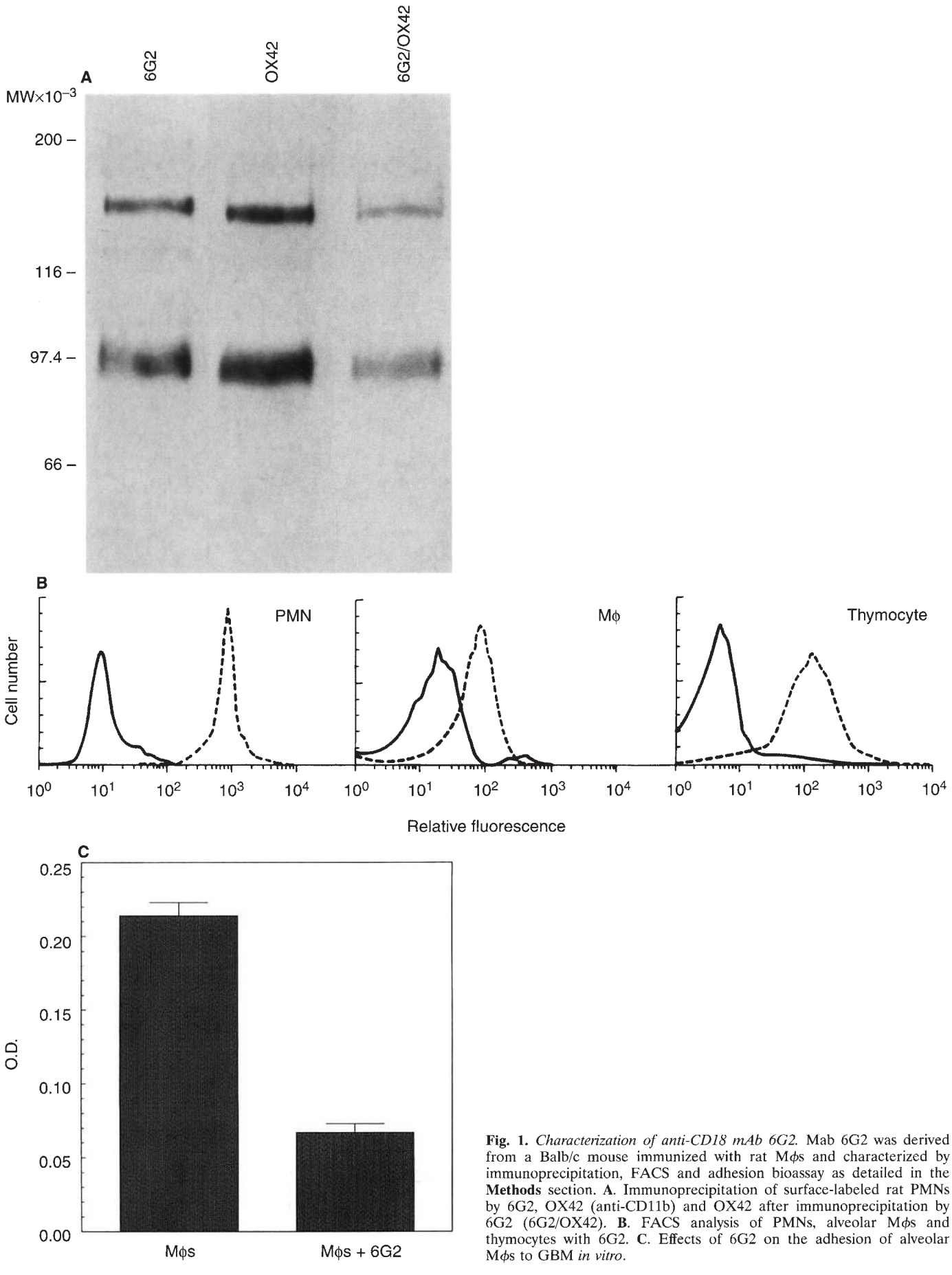

Fig. 1. *Characterization of anti-CD18 mAb 6G2.* Mab 6G2 was derived from a Balb/c mouse immunized with rat Mϕs and characterized by immunoprecipitation, FACS and adhesion bioassay as detailed in the **Methods** section. **A**. Immunoprecipitation of surface-labeled rat PMNs by 6G2, OX42 (anti-CD11b) and OX42 after immunoprecipitation by 6G2 (6G2/OX42). **B**. FACS analysis of PMNs, alveolar Mϕs and thymocytes with 6G2. **C**. Effects of 6G2 on the adhesion of alveolar Mϕs to GBM *in vitro*.

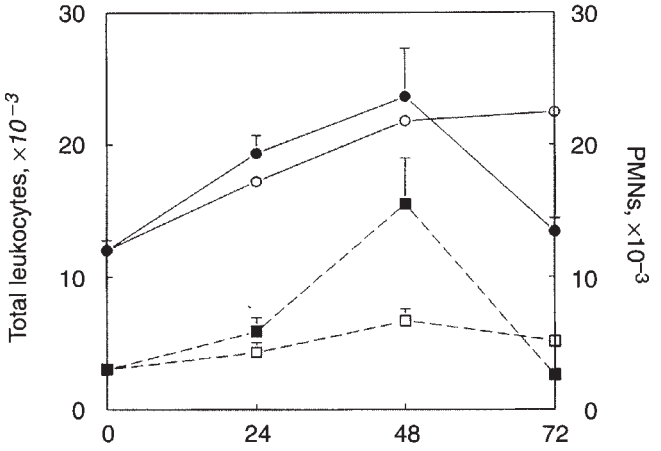

Fig. 2. *Effect of anti-CD18 and -VLA-4 mAbs on circulating leukocytes.* Peripheral leukocyte counts and differential analysis were performed on animals injected with either anti-CD18 (● total; ■ PMNs) or anti-VLA-4 (○ total; □ PMNs) mAb (1 mg i.v.) at selected time points. $N = 3$ for all groups. Circulating leukocytes were elevated at 24 and 48 hours with anti-CD18 mAb treatment, and at all time points with anti-VLA-4 mAb treatment ($P < 0.05$). PMN counts were elevated at 48 hours in anti-CD18 and at 48 and 72 hours in anti-VLA-4 mAb treated animals ($P < 0.05$).

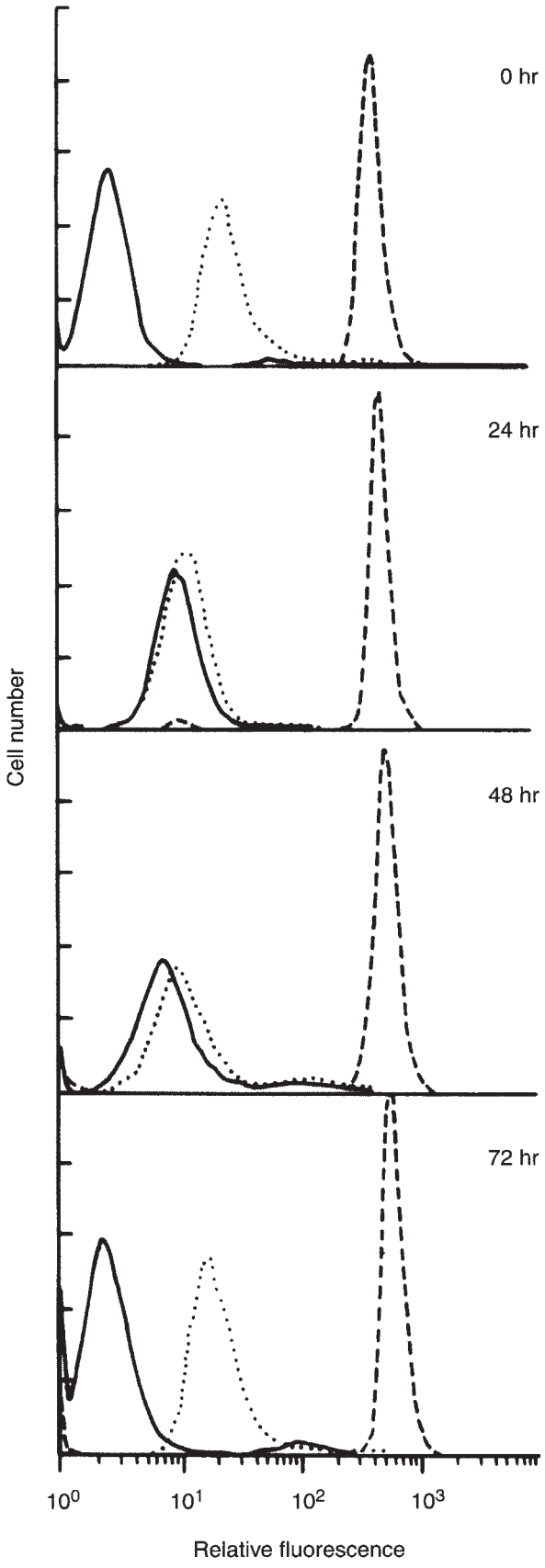

Fig. 3. *In vivo surface saturation of circulating leukocytes after injection of anti-CD18 mAb.* FACS analysis of peripheral leukocytes was performed at varying time points after the injection of anti-CD18 mAb, 6G2, as detailed in the **Methods** section. Solid lines represent labeling *in vivo* (no primary antibody added *in vitro*), while dotted lines represent labeling after exposure to 6G2 *in vitro*. Dashed lines show labeling of leukocytes by CD45.

Methods

Production, characterization and in vivo validation of mAbs to rat CD18 and VLA-4

A mAb of rat CD18 was produced for these studies by immunizing Balb/c mice with rat alveolar Mϕs (Lewis strain, Charles River, Wilmington, MA, USA). The spleen from a mouse with high titer anti-Mϕ reactivity in serum (as determined by immunocytochemisty [16]) was then fused with SP2/0 myeloma cells in the Washington University Hybridoma Center. The resulting clones were then screened for the ability to inhibit Mϕ adherence and spreading on GBM coated-plastic 96 well ELISA plates (Costar, Cambridge, MA, USA). GBM was isolated as published [24]. The clone which was most active in this assay, 6G2, was then further characterized by immunoprecipitation, immunofluorescent staining of leukocytes, and functional assays. Isotype characterization (using a kit from BioRad, Hercules, CA, USA) showed this mAb to be an IgG1.

For immunoprecipitation, PMNs (isolated by peritoneal lavage from rats injected i.p. with 1% glycogen in PBS) were suspended in 0.15 M NaCl, 0.1 M HEPES pH 8.0 containing 1 mg/ml of NHS-LC biotin (Pierce, Rockford, IL, USA). After one hour at room temperature, the cells were washed and then lysed in 150 mM NaCl, 50 mM Tris, 1 mM EDTA, 1% Triton X-100 with protease inhibitors (iodoacetamide 10 mM, leupeptin 10 μg/ml, aprotinin 10 μg/ml, PMSF 1 mM; Sigma, St. Louis, MO, USA).

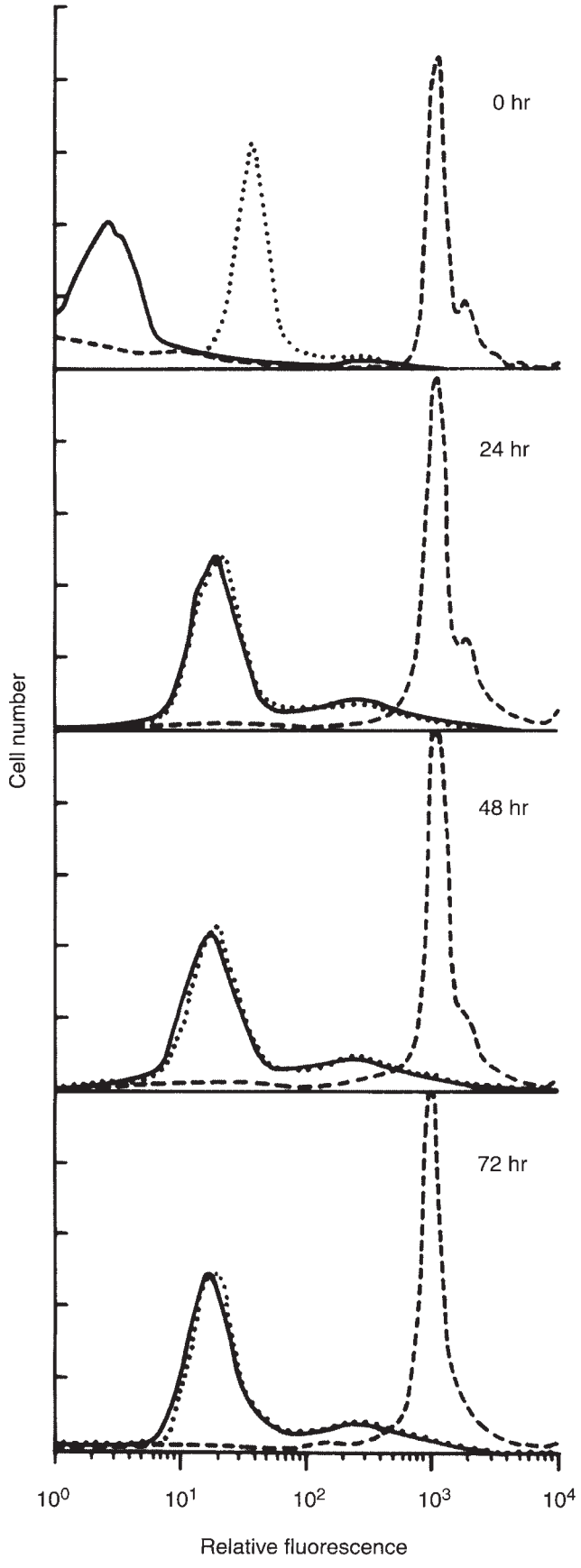

Fig. 4. *In vivo surface saturation of circulating leukocytes after injection of anti-VLA-4 mAb.* FACS analysis of circulating leukocytes was performed at varying time points after the injection of anti-VLA-4 mAb, TA-2, as detailed in methods. Solid lines represent labeling *in vivo*, while dotted lines represent labeling after exposure to TA-2 *in vitro*. Dashed lines show labeling of leukocytes by CD45.

After preclearing, the lysate was immunoprecipitated using 6G2 or OX42 (anti-CD11b mAb [25, 26]), either alone or after 6G2 and agarose anti-mouse IgG (Sigma). Labeled proteins were resolved by SDS-PAGE (7.5% gel) followed by transblotting. Blots were blocked with 10% milk in PBS-0.1% Tween, incubated with avidin-peroxidase (Sigma) diluted 1:1000 vol/vol in PBS-0.1% Tween, and biotin-labeled proteins visualized by chemiluminescence (ECL system; Amersham, Buckinghamshire, UK). FACS analysis was performed as previously described using a rabbit anti-mouse IgG-FITC conjugate (Sigma) employing a FACScan (Becton Dickinson, Mountain View, CA, USA). Alveolar Mϕs, elicited PMNs and thymocytes were used for FACS characterization. Functional assays used for characterization were adherence to plastic [27] and homologous aggregation of thymocytes [28], due to CD11b/CD18 and CD11a/CD18, respectively.

The mAb 6G2 was partially purified prior to use *in vivo*. Ascites containing 6G2 was treated with caprylic acid and the resulting supernatant filtered through a Centricon 100 (Amicon, Beverly, MA, USA). Antibody was then suspended in sterile 0.9% NaCl, sterile filtered, and stored at −20°C prior to use at 1 mg/ml. Endotoxin content of this antibody was < 200 pg/ml.

Development and characterization of the mAb to rat VLA-4, TA-2, have been published previously [29]. This mAb is an IgG1 that recognizes the CD49d subunit and is function-blocking. TA-2 was used as sterile ascites diluted to an antibody content of 2 mg/ml. As a control for mAb administration, B9, an IgG1 mAb to pertussis toxin, was given to a group of nephritic animals (also as sterile ascites diluted to 2 mg/ml) [30]. Administration of the control mAb, B9, did not alter the leukocyte influx or proteinuria in anti-GBM nephritis (data not shown). In consequence, data from B9-treated and untreated controls were combined.

MAbs were administered to animals by intravenous injection. In order to validate efficacy of adhesion receptor blockade, coating of circulating leukocytes by antibody was determined at 0, 24, 48, and 72 hours after injection of mAb by flow cytometry using previously detailed methods [16]. In brief, peripheral leukocytes were isolated from heparinized blood which was adjusted to 1% dextran by sedimentation at 1 g for one hour at 25°C. Leukocytes were then labeled with a rabbit anti-mouse IgG F(ab')-FITC conjugate (Sigma) directly to assess the number of cells labeled with the mAb *in vivo*. Alternatively, leukocytes were exposed to the same mAb as that injected intravenously prior to labeling to assess the total number of cells expressing the relevant adhesion receptor. Comparison of these two curves was used to determine the efficiency of *in vivo* adhesion receptor blockade. As a control and to aid in gating, peripheral leukocytes were also labeled for CD45 using OX1 (Chemicon, Temecula, CA, USA). Labeled cells were quantified on a FACScan as above. In parallel experiments, circulating leukocyte counts and differential analysis were performed by hemocytometer and by using Diff-Quick stained blood films (Baxter, McGaw Park, IL, USA).

Induction and manipulation of heterologous anti-GBM nephritis

Heterologous anti-GBM nephritis was induced in Lewis rats (Charles River) by the intravenous injection of a rabbit polyclonal antibody to rat GBM described previously [16]. This model of nephritis is characterized by linear deposition of IgG and C3 in the GBM, accompanied by an infiltrative/diffuse proliferative nephritis with proteinuria lasting > 72 hours [reviewed in 1, 2]. To assess proteinuria, urine was collected using a metabolic cage and protein determined by the Bradford assay (Bio-Rad). To quantify the influx of leukocytes into the glomerulus, glomeruli were isolated from saline-perfused kidneys using a sieving protocol and dispersed into a single cell preparation as previously described [16]. Cells were centrifuged onto slides (Shandon Inc., Pittsburgh, PA, USA), fixed with methanol/acetone (50:50 vol/vol), and leukocytes were labeled with an anti-CD45 antibody (OX1) as detailed [16]. Hematoxylin was used as a counterstain. The labeled cells were quantified as a percentage of the total by light microscopy and categorized by nuclear morphology as to lineage. Total leukocyte content was calculated by multiplying percentages by the cell yield per glomerulus. This method correlates well with other methods of leukocyte quantification (staining tissue sections, *in situ* labeling of intact glomeruli) [31, 32].

To study the effect of mAbs on anti-GBM nephritis, rats were treated with 1 mg of mAb (6G2, anti-CD18; TA-2, anti-VLA-4/CD49d; or B9, control mAb) 30 minutes prior to induction of disease and in some experiments 36 hours after disease induction. To assess the effect on the early inflammatory phase, proteinuria was determined by collecting urine from 0 to 24 hours after disease induction and glomerular leukocytes quantified at three hours after disease induction. To assess the effect on the later inflammatory phase, proteinuria was determined by collecting urine from 48 to 72 hours after disease induction and glomerular leukocytes quantified at 72 hours.

To determine the effects of mAbs on Mϕ migration/activation in the absence of acute inflammation, animals were either complement- or PMN-depleted prior to induction of nephritis. Complement depletion was effected using cobra venom factor (CVF) as previously described (CVF; Diamedix, Miami, FL, USA) [33]. PMN depletion was accomplished using a rabbit anti-rat PMN antibody (Accurate; Westbury, NY, USA) as published [23]. PMN depletion was induced before the induction of disease by the injection of anti-PMN antiserum (2.5 ml/kg). To determine the effect of CD18 and VLA-4 blockade in these animals, mAbs were administered 24 hours after disease induction.

Data analysis

FACS, immunoprecipitation, and adherence bioassay data are presented as representative experiments (1 out of 3). Other data are presented as the mean ± SEM of several independent experiments with the appropriate N indicated in the Figure legends or text. Because substantial interexperimental variability in proteinuria was observed (in part due to the use of different batches of anti-GBM antiserum), proteinuria is normalized to contemporaneously performed controls for statistical comparison. Quantitative data on proteinuria (averages and ranges), however, are also presented in the text or Figure legends. Less variability in glomerular leukocyte counts was observed (since experiments were performed with a single batch of anti-GBM) and non-normalized data are presented. Data were analyzed by ANOVA using Bonferroni's correction for multiple comparisons and statistical significance is indicated if $P < 0.05$.

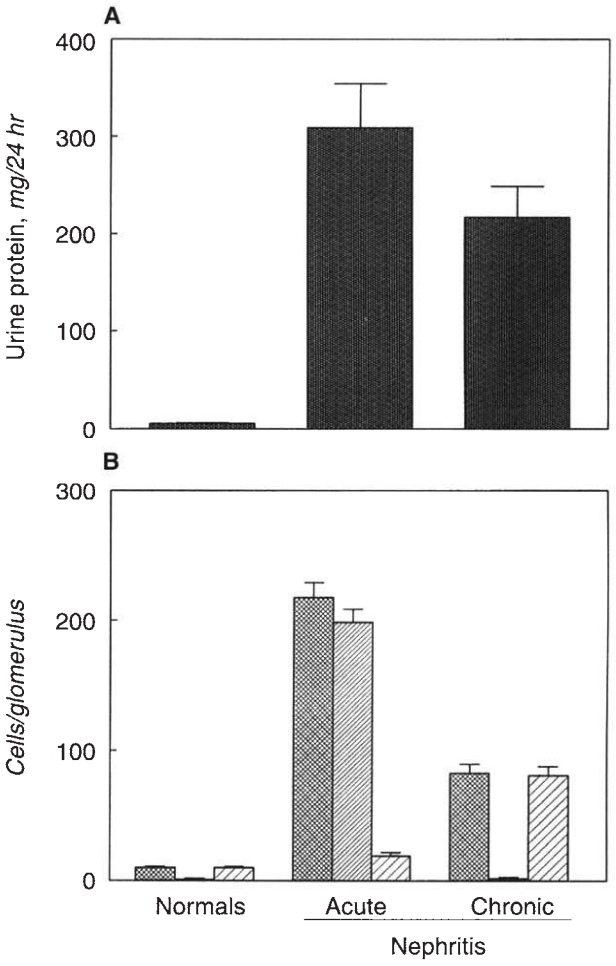

Fig. 5. *Evolution of proteinuria and glomerular leukocyte counts during acute and chronic phases of heterologous anti-GBM nephritis.* Animals were injected with anti-GBM antibody and proteinuria determined during the acute (0 to 24 hr) and chronic phases (48 to 72 hr) of the disease model (**A**). Glomeruli from nephritic animals were also removed during the acute and chronic phases (3 and 72 hr, respectively) and glomerular leukocytes quantified and subtyped by immunocytochemistry as detailed in the **Methods** section (**B**). The data shown are the aggegate quantitative data from the controls used in the experiments shown in Figures 6 to 9 ($N = 8$). Data from normal animals are provided for comparison ($N = 10$). Symbols are: (▩) total; (▨) PMNs; (▨) Mϕs.

Results

Characterization of 6G2 as an anti-CD18 mAb

The mAb 6G2 was characterized as directed against CD18 by immunoprecipitation, FACS and adherence bioassay. As shown in Figure 1A, mAb 6G2 immunoprecipitated a dimer from PMNs which comigrated with that immunoprecipitated by OX42, CD11b/CD18 (Fig. 1A). Preclearing lysates with 6G2 substantially diminished the intensity of the bands immunoprecipitated by OX42 indicating that 6G2 recognized CD11b/CD18 (Fig. 1A). Variably, an additional band below the CD11b band was immunoprecipitated from PMNs with 6G2 (presumably CD11c, data not shown). Both myeloid cells (PMNs, Mϕs) and lymphoid cells

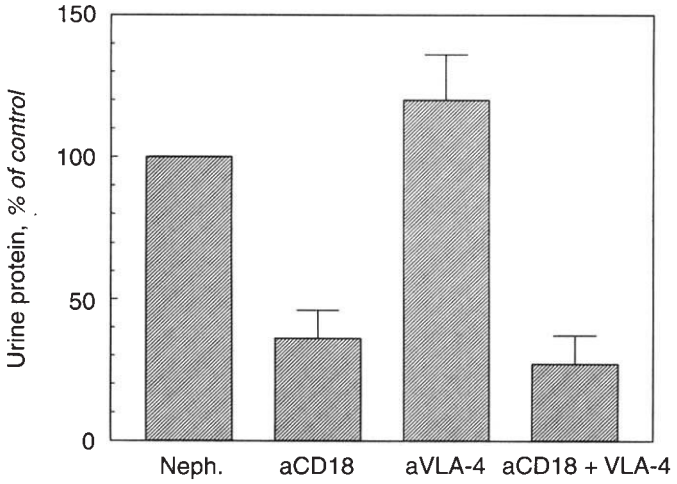

Fig. 6. *Effect of mAbs to CD18 and VLA-4 on proteinuria from 0 to 24 hours after induction of anti-GBM nephritis.* Proteinuria was measured over the initial 24 hours of disease in nephritic animals (Neph.) and groups of nephritic animals given anti-CD18 and/or anti-VLA-4 mAbs 30 minutes prior to disease induction (αCD18, αVLA-4, and αCD18+VLA-4, respectively). Proteinuria was normalized to contemporaneously performed controls (= 100%). N = 8, 6, 3, and 5 in the groups shown. The decrease in proteinuria in animals given anti-CD18 mAb (alone or in combination with anti-VLA-4) relative to control was statistically significant ($P < 0.05$).

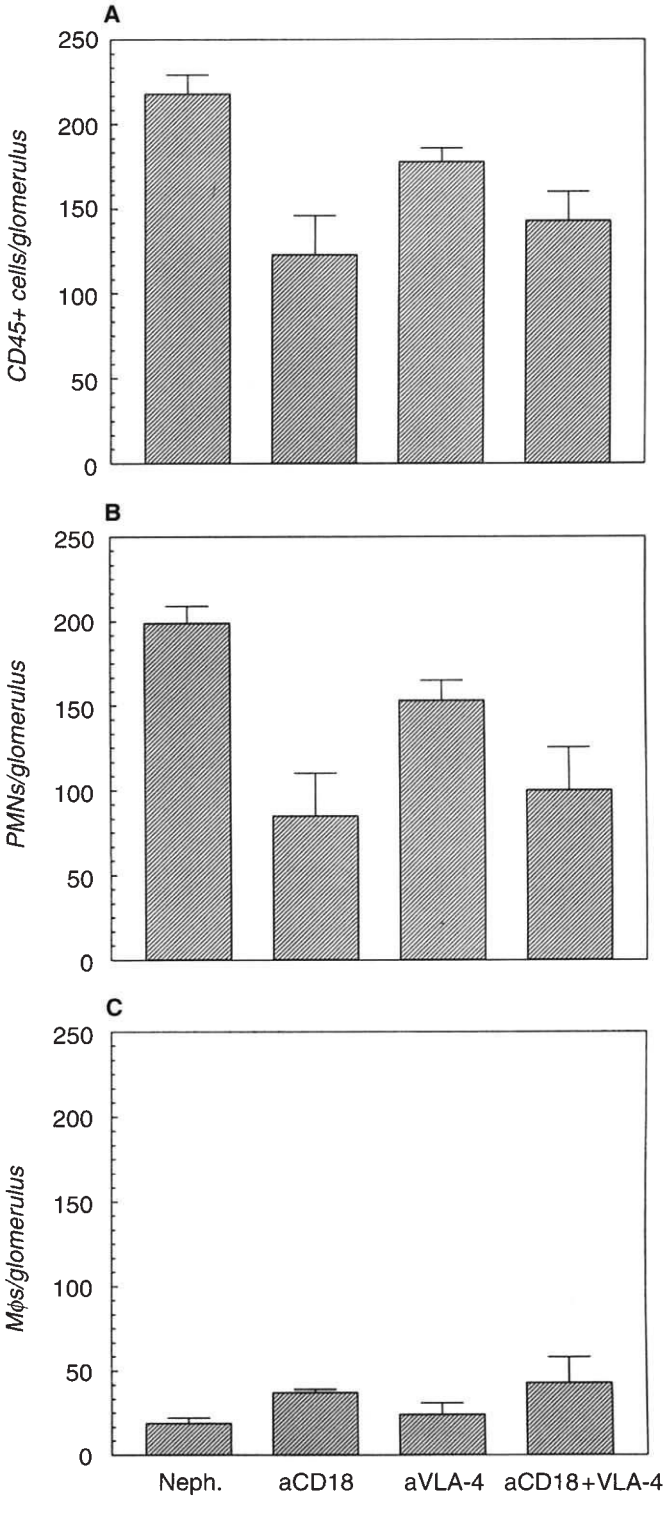

Fig. 7. *Effect of mAbs to CD18 and VLA-4 on glomerular leukocytes three hours after induction of anti-GBM nephritis.* Glomerular leukocytes were measured by immunocytochemistry (**Methods**) three hours after induction of nephritis in controls (Neph.), anti-CD18 mAb pretreated animals, anti-VLA-4 mAb pretreated animals, and animals pretreated with combination therapy (αCD18, αVLA-4, and αCD18+VLA-4, respectively). Total glomerular leukocytes (**A**) as well as PMN (**B**) and Mϕ (**C**) counts are shown. N = 4, 4, 3, and 3, respectively, for the groups as shown. Glomerular leukocytes were decreased significantly in anti-CD18 mAb treated animals (alone or in combination, $P < 0.05$). There was a trend towards significance for anti-VLA-4 treated animals ($P = 0.09$) For PMNs, all three experimental groups were significantly decreased relative to control ($P < 0.01$ for anti-CD18-treated animals, $P < 0.05$ for anti-VLA-4 treated animals).

(thymocytes) exhibited the antigen recognized by 6G2 (Fig. 1B), whereas OX42 labeled only myeloid cells ([25] and data not shown). Also, as shown in Figure 1C, 6G2 was function-blocking in that it inhibited Mϕ adherence to tissue culture plastic *in vitro*. Homologous aggregation of thymocytes was also blocked with 6G2 (data not shown). On the basis of these results, we concluded that 6G2 recognized CD18.

In vivo validation of mAb blockade of CD18 and VLA-4

The *in vivo* effect of the mAbs, 6G2 (anti-CD18) and TA-2 (anti-VLA-4/CD49d), on peripheral leukocyte counts and *in vivo* saturation of leukocyte adhesion molecules was determined subsequently. *In vivo* administration of either mAb alone (or both mAbs together) produced a leukocytosis which lasted for > 48 hours for 6G2 (anti-CD18) and > 72 hours for TA-2 (anti-VLA-4/CD49d) (Fig. 2). For 6G2 (anti-CD18), the leukocytosis was predominantly due to an increase in PMNs, while the leukocytosis due to TA-2 (anti-VLA-4/CD49d) resulted more from an increase in lymphocytes (Fig. 2). Combination blockade produced an additive effect (data not shown). By FACS analysis, 6G2 both down-regulated the expression of CD18 by peripheral leukocytes and effectively saturated CD18 for ca. 48 hours (Fig. 3). The *in*

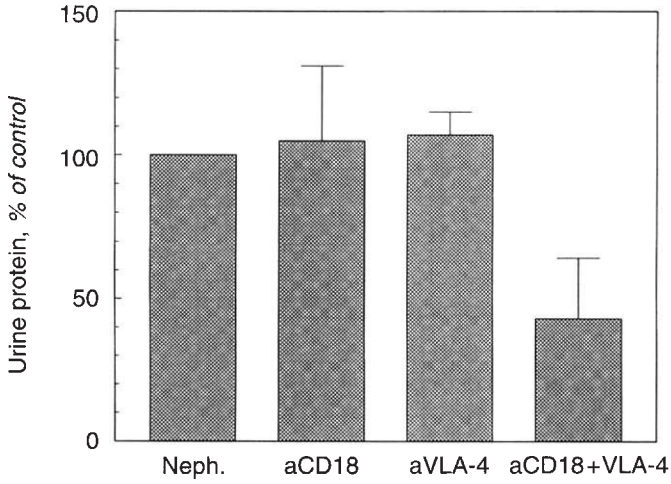

Fig. 8. *Effect of mAbs to CD18 and VLA-4 on proteinuria from 48 to 72 hours after induction of anti-GBM nephritis.* Proteinuria was measured in nephritic animals (Neph.) and subgroups of nephritic animals given anti-CD18 and/or anti-VLA-4 mAbs 30 minutes prior to, and 36 hours after, disease induction (anti-CD18, anti-VLA-4, and anti-CD18+VLA-4, respectively). Proteinuria was normalized to contemporancously performed controls (= 100%). N = 8, 6, 3, and 5, respectively, in the groups shown. The decrease in proteinuria in animals given anti-CD18 and anti-VLA-4 mAbs together relative to control was statistically significant ($P < 0.05$).

vivo effects of TA-2 were similar except that the duration of blockade was even more prolonged with effective saturation of leukocyte VLA-4 observed up to 72 hours (Fig. 4).

Effects of anti-CD18 and anti-VLA-4 mAbs on the early phase of heterologous anti-GBM nephritis

The early phase of heterologous anti-GBM nephritis was characterized by proteinuria which occurred concomitantly with an influx of PMNs into the glomerulus (Fig. 5). We initially determined the effects of mAb blockade of leukocyte adhesion receptors on this phase of the disease model by pretreating animals the aforementioned mAbs prior to disease induction. Although there was variability in the acute proteinuria between experiments, proteinuria in nephritic animals averaged 309 ± 45 mg/24 hr (range 166 to 615 mg/24 hr), indicating severe glomerular injury (5 ± 1 mg/24 hr in normal animals). Anti-CD18 mAb significantly reduced the early phase proteinuria (0 to 24 hr) by ca. 60% whereas anti-VLA-4 mAb was ineffective (Fig. 6). Combination blockade of CD18 and VLA-4 was no more effective than anti-CD18 blockade alone in reducing proteinuria.

The decrease in proteinuria seen with mAb to CD18 was accompanied by a significant reduction in glomerular leukocytes due principally to a reduction in the influx of PMNs (Fig. 7). The decrease in PMNs with CD18 blockade was commensurate to the decrease in proteinuria (both ca. 60%). Although mAb to VLA-4 did not effect proteinuria, a small reduction in glomerular leukocyte counts was observed due to a decrease in the influx of PMNs (Fig. 7). The combination of mAbs to CD18 and VLA-4, however, were not additive. Glomerular Mϕs were detectible (but not prominent) at this time point; however, their numbers were not altered by mAb administration (Fig. 7).

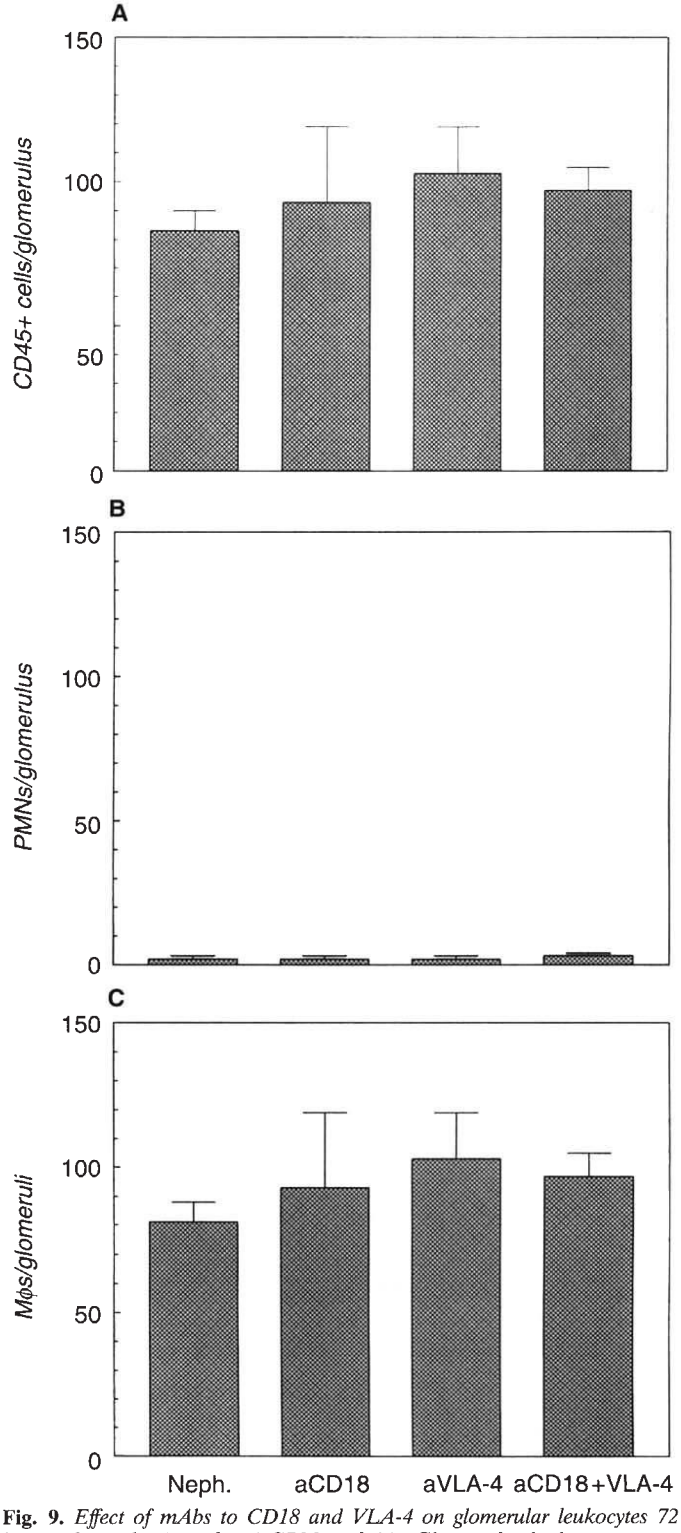

Fig. 9. *Effect of mAbs to CD18 and VLA-4 on glomerular leukocytes 72 hours after induction of anti-GBM nephritis.* Glomerular leukocytes were measured by immunocytochemistry (**Methods**) 72 hours after induction of nephritis in controls (Neph.), anti-CD18 mAb treated animals (anti-CD18), anti-VLA-4 mAb treated animals (anti-VLA-4), and animals treated with combination therapy (anti-CD18+VLA-4). MAb therapy was administered 30 minutes prior to, and 36 hours following, disease induction. Total glomerular leukocytes (**A**) as well as PMN (**B**) and Mϕ (**C**) counts are shown. N = 12, 4, 3, and 5, respectively, for the groups as shown. No statistically significant changes were observed.

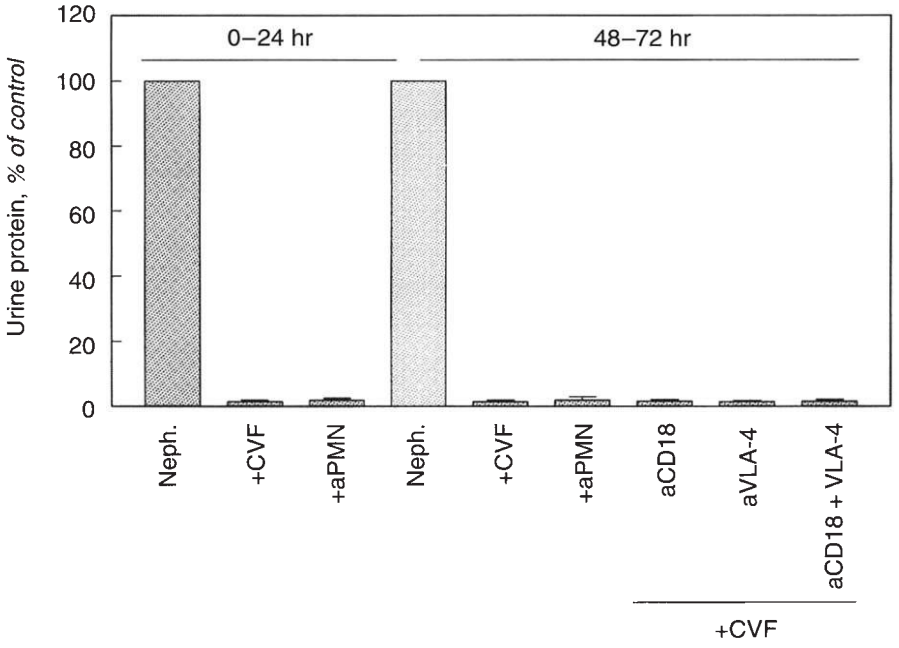

Fig. 10. *The effect of complement and PMN depletion on proteinuria after induction of anti-GBM nephritis.* Nephritis was induced in controls (Neph.), or animals who had been depleted of complement or PMNs prior to disease induction using CVF or a specific polyclonal antiserum (αPMN). Subgroups of decomplemented animals also received mAb to CD18, VLA-4 or both 24 hours after disease induction (CVF+αCD18, CVF+αVLA-4, CVF+αCD18+VLA-4). Proteinuria was measured from 0 to 24 hours and from 48 to 72 hours and is normalized to contemporaneously performed controls. Average proteinuria in controls was 258 ± 34 mg/24 hours (range 100 to 517 mg/24 hr) from 0 to 24 hours and 118 ± 20 mg/24 hours (range 40 to 252 mg/24 hr) from 48 to 72 hours. N = 11, 18, 3, 11, 5, 3, 6, 4, and 3 in the groups shown. The decreases in proteinuria observed at both 0 to 24 and 48 to 72 hours in all experimental groups relative to control were statistically significant ($P < 0.001$).

Effects of anti-CD18 and anti-VLA-4 mAbs on the later phase of heterologous anti-GBM nephritis

Proteinuria persisted during the later phase of heterologous anti-GBM nephritis as the leukocyte infiltrate became exclusively comprised of Mϕs; however, the average proteinuria was still substantial: 217 ± 32 mg/24 hr (range 82 to 469 mg/24 hr) (Fig. 5). In order to assess the effect of CD18 and/or VLA-4 blockade on this phase of heterologous anti-GBM nephritis, mAbs were readministered to animals 36 hours after disease induction based on the aforementioned *in vivo* validation data (see above). Despite a substantial attenuation of the early phase proteinuria (0 to 24 hr), continued blockade of CD18 did not attenuate the proteinuria occurring between 48 and 72 hours after disease induction (Fig. 8). VLA-4 blockade alone also had no significant effect on proteinuria (Fig. 8). Combination blockade, however, did significantly diminish proteinuria (ca. 50%) although substantial proteinuria was still observed in animals treated with combination therapy (Fig. 8).

In accord with the proteinuria data, neither blockade of CD18 nor VLA-4 altered the influx of glomerular leukocytes during the later phase of anti-GBM nephritis which are almost exclusively Mϕs (Fig. 9). Although combination blockade attenuated proteinuria (Fig. 8), no significant effect was observed on glomerular leukocyte influx (Fig. 9).

Effects of anti-CD18 and anti-VLA-4 mAbs on the later phase of heterologous anti-GBM nephritis in the absence of acute inflammation

Because Mϕ migration into inflamed glomeruli has been shown to occur independently of the influx of PMNs [19, 20], we also addressed the effects of mAbs to CD18 and VLA-4 on the influx of Mϕs into glomeruli in animals in whom the early inflammatory phase of anti-GBM nephritis was blocked either by complement or PMN depletion. As we have previously observed, both complement and PMN depletion completely blocked the early proteinuria in this model (Fig. 10) [23, 33]. This decrease in proteinuria is due to an absence of PMN infiltration into glomeruli ([23, 33] and data not shown). Although PMN migration is abrogated by these manipulations, Mϕ migration was not altered, although the proteinuria which typically accompanies the influx of Mϕs was completely blocked (Figs. 10 and 11). This diminished influx of Mϕs could not be attributed to the lack of complement since CVF and PMN depletion produced identical results (Figs. 10 and 11).

Because proteinuria in decomplemented animals was already prevented, mAb administration had no further effect (Fig. 10). However, we did observe that mAb to VLA-4, but not CD18, significantly inhibited the influx of Mϕs in response to the deposition of anti-GBM antibody by ca. 50% (Fig. 11). Additionally, combining both CD18 and VLA-4 mAbs was no more effective than mAb to VLA-4 alone (Fig. 10). The effect of the mAbs was identical in PMN-depleted animals (data not shown).

Discussion

The current study provides several important insights into the mechanisms underlying the influx of leukocytes into inflamed glomeruli during the course of glomerulonephritis. With respect to acute inflammation, this study establishes the importance of CD18 to PMN migration and activation in acute nephritis. As we observed, PMN migration into inflamed glomeruli and *in situ* activation were blocked ca. 60% by saturating levels of anti-CD18 mAb. This degree of inhibition, though, is intermediate relative to other models of inflammation [14, 15], and supports the concept that the contribution of CD18 to PMN migration/activation may vary substantially depending on the inflammatory stimulus or tissue involved.

The data also establish that there is a significant CD18-independent component to these processes. The presence of such a CD18-independent pathway may explain why anti-CD18 mAbs have not been invariably effective in inhibiting acute nephritis

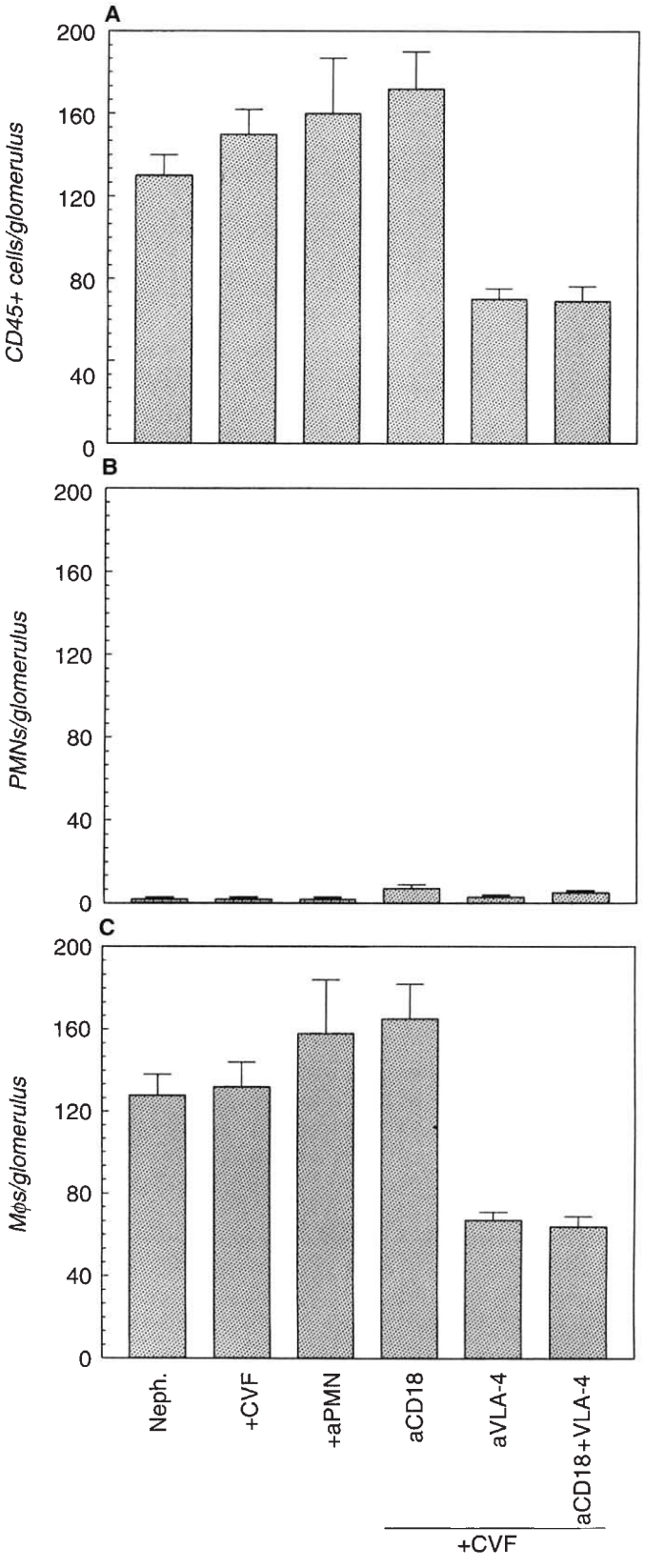

Fig. 11. *Effect of mAbs to CD18 and VLA-4 on glomerular leukocytes 72 hours after induction of anti-GBM nephritis after abrogation of acute inflammation.* Glomerular leukocytes were measured by immunocytochemistry (**Methods**) 72 hours after induction of nephritis in controls (Neph.), decomplemented animals (+CVF), PMN-depleted animals (αPMN), and decomplemented animals treated with mAbs to either CD18, VLA-4 or both (CVF+αCD18, CVF+αVLA-4, CVF+αCD18+VLA-4). MAb therapy was administered 24 hours following disease induction. Total glomerular leukocytes (**A**) as well as PMN (**B**) and Mϕ (**C**) counts are shown. $N = 10, 3, 3, 5, 5$, and 3, respectively, for the groups as shown. Decomplemented animals treated with anti-VLA-4 mAb (alone or in combination) exhibited a decrease in glomerular leukocytes and Mϕs relative to control ($P < 0.01$).

[18]. Although prior studies have suggested that CD18-independent PMN migration/activation may involve VLA-4 [11], this conclusion does not appear applicable to this model of nephritis. Although this study, and a prior one [17], noted a modest effect of VLA-4 blockade on PMN migration/activation, the current work demonstrates that this effect is not additive to that of CD18 blockade. The nature of the CD18-independent pathway for PMN migration/activation in this model of inflammation thus remains to be clarified.

With respect to the later phase of heterologous anti-GBM nephritis, the present study suggests that the *in vivo* migration of monocytes into inflamed glomeruli is more complex than what has been observed for monocyte adherence and transendothelial migration *in vitro* [7–10] and monocyte migration *in vivo* in other models of inflammation [11–13]. In these cited studies, monocyte adherence and migration appear to be largely mediated through a cooperation between CD18 and VLA-4 [7–10]. In the present study, however, we found that CD18 did not contribute significantly to monocyte migration into inflamed glomeruli *in vivo*. However, VLA-4 did appear to mediate a component of monocyte migration into inflamed glomeruli, although its contribution in this regard was diminished if preceded by a severe acute inflammatory phase. With respect to monocyte/Mϕ activation, the current data suggest that both VLA-4 and CD18 may make a contribution although we cannot dissect out the individual contributions of each from the current data.

The relevance of our findings to the chronic phase of anti-GBM nephritis (which may progress to glomerulosclerosis) remains to be clarified. Apropos to this issue, we would note that recent studies on the evolution of chronic anti-GBM nephritis found that CD11a/CD18 and its counterreceptor, ICAM-1, were cooperatively important for the development of chronic nephritis and glomerulosclerosis, potentially via a decrease in Mϕ migration and activation [21, 22]. There are several possible reasons for the apparent difference between these studies and the current one regarding the role of CD18 in nephritis. First, chronic nephritis requires an active immune response (that is, antibody formation) whereas the heterologous phase of anti-GBM nephritis is a passive model. Second, chronic nephritis is often characterized by the accumulation of glomerular T cells (which may be blocked by anti-CD18 therapy), whereas in the heterologous phase of anti-GBM nephritis lymphocytes are typically absent [34]. Third, a contribution of PMNs to the glomerular injury in the cited studies was not conclusively ruled out. Although CD11a/CD18 does not appear to contribute PMN migration and activation in nephritis, ICAM-1 does make a major contribution through its interaction with CD11b/CD18 [17]. Fourth, Mϕ migration and activation in chronic nephritis may involve distinct mechanisms from those engaged earlier on in disease pathogenesis.

With respect to this latter issue, we would note that while our data demonstrate that Mϕ migration in heterologous anti-GBM nephritis is PMN- and complement-independent, we found that Mϕ activation (in terms of proteinuria) is not. In fact, Mϕ migration into glomeruli in heterologous anti-GBM nephritis was not associated with proteinuria in the absence of a preceding acute inflammatory response. In contrast, both Mϕ migration and activation are PMN- and complement-independent in chronic anti-GBM nephritis [19, 20]. These observations support the above hypothesis that the mechanisms underlying Mϕ migration and activation in chronic autologous anti-GBM nephritis may differ from those in the more acute phase of this disease model. Regardless, we have observed that PMNs may make an important contribution to chronic glomerular injury and glomerulosclerosis independently of Mϕs [35].

In sum, the current study establishes that both CD18 and VLA-4 contribute to leukocyte migration and activation in heterologous anti-GBM nephritis, although there are clearly important non-CD18/VLA-4 pathways that participate in these process. The contribution of CD18 and VLA-4 to chronic nephritis and glomerular scarring, however, remain to be more clearly defined. Further clarification of these issues may help elucidate further how leukocyte migration/activation can be manipulated to ameliorate glomerular dysfunction and its long term consequence of glomerulosclerosis. Given the limited pharmacologic options currently available for treating immune-mediated glomerulonephritis in humans, such studies may have potential therapeutic impact.

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Appendix

Abbreviations are: PMN, polymorphonuclear neutrophil; Mϕ, macrophage; GBM, glomerular basement membrane; mAb, monoclonal antibody.

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